P-29

48851

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Figure: 2

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Awards Abstract

Kinetic Tetrazolium Microtiter Assay

Microbial contamination and the resistance of cells to disinfectants in potable water systems of the Space Transportation System (STS) are currently assessed terrestrially by isolating the bacteria using membrane filtration or tube dilution techniques. Such methods are too slow and difficult, especially in microgravity conditions. For longer duration space travel and habitation, as well as numerous terrestrial applications, it is important to have the capability to self-monitor water system potability. Therefore a need exists for a rapid, quantitative, statistically valid *in vitro* bacterial assay. Several methods have been developed to assay cell growth of bacterial and mammalian cells. Such methods, particularly the standard plate count and tube dilution methods, are lengthy, labor intensive and the results are frequently statistically invalid because of the standard error in comparing trial repetitions. Plate counts often do not produce viable colonies due to the inability of the cells to proliferate under a confined class of culture conditions. In addition, microbial clumping can lead to underestimation of bacterial density. Clumping can also occur in tube dilutions. Both tube and plate methods are cumbersome and require a large number of dilutions, plates and tubes.

The invention relates to a tetrazolium cell assay method wherein tetrazolium reduction products are dispersed *in vitro* using a nonionic detergent. The *in vitro* cell assay method uses tetrazolium as an indicator. The indicator includes a nonionic detergent which solubilizes a tetrazolium reduction product *in vitro* and has low toxicity for the cells. The incubation of test cells in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and octoxynol (TRITON X-100) permits kinetics of the cell metabolism to be determined.

Novelty exists in a tetrazolium assay method (which is surprisingly without substantial effect on the cell growth) which permits rapid measurement of persistent cell growth in response to growth or cytotoxic factors. The method couples the cellular reduction of a tetrazolium salt with an *in vitro* detergent which solubilizes the formazan product, permitting direct spectrophotometric measurement of the formazan as it is produced and the determination of cell kinetics.

Inventors: Duane L. Pierson*, Raymond P. Stowe**, David W. Koeing**

Employer: Johnson Space Center* and KRUG Life Sciences, Inc.**

Evaluator: Helen W. Lane

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KINETIC TETRAZOLIUM MICROTITER ASSAY

Origin of the Invention

The invention described herein was made in the performance of work under a NASA contract and is subject to the provisions of Section 305 of the National Aeronautics and Space Act of 1958, Public Law 85-568 (72 Stat. 435; 42 U.S.C. 2457).

Field of the Invention

The present invention relates to a method for conducting a cell assay using a tetrazolium compound. More particularly the present invention relates to a tetrazolium assay method wherein tetrazolium reduction products are dispersed *in vitro* using a nonionic detergent.

Background of the Invention

Several methods have been developed to assay cell growth of bacterial and mammalian cells. Important applications include screening antimicrobial and anticancer agents for efficacy and detection of pathogens in water treatment and biologic fluids. Among commonly used methods are tritiated thymidine uptake as described in Twentyman, P. R., et al., British Journal of Cancer, 50:625, 1984; dye exclusion as described in Wiesenthal, L. M., et al., Cancer Research, 43:749, 1983; standard plate count or tube dilation described by American Public Health, 17th ed., Washington D. C.: American Public Health Association, 1989; and turbidimetric measurement as described in Collins, C. H., et al., Microbiological Methods, Boston: Butterworth, 1989, pp. 127-140. Such methods, particularly the standard plate count and tube dilution methods, are lengthy, labor intensive and the results are frequently statistically invalid because of the standard error in comparing trial repetitions. Plate counts often do not produce visible colonies due to the

inability of the cells to proliferate under a confined class of culture conditions. In addition, microbial clumping can lead to underestimation of bacterial density. Clumping can also occur in tube dilutions. Both tube and plate methods are cumbersome and require a large number of dilutions, plates and tubes.

One method known for the rapid assessment of cell growth is tetrazolium reduction assay. Colorless tetrazolium salts are reduced in the cell by action of the enzyme dehydrogenase to form a corresponding formazan dye compound. Since this conversion appears to be universal in growing cells, the amount of formazan present is generally proportional to the number of cells in a culture. The formazan concentration can be measured *in situ* by well known spectrophotometric techniques. Thus, this method can be used to enumerate the growth of microorganisms and mammalian cells in microplate cytotoxicity assays. Microorganism tetrazolium assay is described in Bartlett, et al. Journal of Clinical Microbiology, 3(3):327-329, 1976; Jeffrey, et al. Applied Environmental Microbiology, 51(1):150-156, 1986; Levitz, et al., Journal of Infectious Diseases, 152(5):938-945, 1985; and EPA 0,322,591, published May 7, 1989. Mammalian cell assay is described in Alley, et al., Proceedings of the American Association of Cancer Research, 27:389, 1986; Carmichael, et al., Cancer Research, 47:936-942, 1987; and Mosmann, Journal of Immunological Methods, 65:55-63, 1983.

Since the formazan compounds produced in the cells are typically insoluble in water, a development step has generally been necessary to obtain color indication. This has usually required the addition of a solvent such as dimethyl sulfoxide (DMSO) or isopropanol. However, such solvents are detrimental to cell growth and generally terminate the culture.

Nonionic detergents have found use in dissolving cell membranes and in solubilizing membrane-bound proteins. In dissolving cell membranes, the detergents

form detergent-lipid and detergent-lipid-protein mixed micelles. The detergents prefer to form micelles rather than bilayers due to geometries involved. Thus, an excess of these compounds, when added to a membrane suspension, will tend to shift the equilibrium of all amphipathic molecules in the mixture from bilayer to mixed micelle. This concentration is known as the critical micelle concentration (CMC). Each detergent has a characteristic CMC above which it exists in aqueous solution almost entirely in the micellar form.

In the case of protein solubilization, the initial detergent action upon the membrane does not involve rupture, but rather, some of the proteins are "extracted" to form protein-soap aggregates. In both instances, hydrophobic elements are bound to nonpolar monomer sites on the detergent molecule. This is further detailed in Lichtenberg et al., Biochimica et Biophysica Acta 737:285-304, 1983 and Helenius et al., Biochimica et Biophysica Acta 415:29-79, 1975. According to Schnaitman, Journal of Bacteriology 108:553-563 and 108:545-552, 1971, bacterial cell walls, even fractured ones, were surprisingly resistant to the action of TRITON X-100 octoxynol detergent.

Microbial contamination and resistance to disinfectants in potable water systems of the space transportation system (STS) are currently assessed terrestrially by isolating the bacteria using membrane filtration or tube dilution techniques. Such methods are too slow and difficult in microgravity conditions. For longer duration space travel and habitation, it will be important to have the capability to self-monitor water system potability. Therefore a need exists for a rapid, quantitative, statistically valid *in vitro* bacterial assay which is conducive for space use.

Additional references of interest include U. S. patents 4,728,608 to Roberts et al.; 4,235,964 to Bochner; 2,967,132 to Sacks and Russian patents 1,564,193 and 1,063,835.

Summary of the Invention

A tetrazolium assay method, which is surprisingly without substantial effect on the cell growth, has been developed to permit rapid measurement of cell growth in response to growth or cytotoxic factors. The method couples the cellular reduction of a tetrazolium salt with an *in vitro* detergent which solubilizes the formazan product. The method permits direct spectrophotometric measurement of the formazan as it is produced and determination of cell kinetics.

In one aspect, the present invention provides a method for conducting a non-destructive tetrazolium assay of cell metabolism. In one step, viable test cells and a suitable growth medium are placed in a plurality of wells. Indicator reagents comprising a tetrazolium compound reduced by cell metabolism to a formazan product, an optional electron carrier for facilitating the tetrazolium compound reduction and a nonionic detergent for dispersing the formazan product are added to the growth medium. The metabolism of the test cells is substantially unaffected by the indicator reagents. The indicator-containing cells are incubated in the presence of the reagents for a time sufficient to induce growth in the test cells and produce the formazan product. The formazan product is dispersed *in vitro* by the nonionic detergent to produce a color change in the test medium. The extent of the color change is determined, and cell growth can be assessed. For kinetic assay, the culture can be incubated further and the color change determination conducted at one or more later intervals.

As a preferred embodiment, the tetrazolium compound comprises 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, the nonionic detergent comprises octoxynol and the electron carrier comprises N-methylphenazonium methosufate. The tetrazolium compound is preferably used in an amount of from about 20 μM to

about 250 μM. The nonionic detergent is preferably used in an amount of from about 1 mM to about 20 mM. The electron carrier is preferably used in an amount of from about 100 μM to about 400 μM.

The method can further include the steps of initially culturing the test cell to a suitable density and adding an effector test agent such as a growth inhibitor or a growth stimulant prior to the incubating step. The cell growth inhibitor can be an antimicrobial or anticancer agent. The test agent can also be a substrate material screened for biodegradability (i. e. capable of sustaining cell growth) or a chemical or waste material screened for biocompatibility (i. e. innocuous to cell growth).

Brief Description of the Figures

Fig. 1 shows a comparison of absorption spectra of *E. coli* formazan produced and developed using an octoxynol surfactant TRITON X-100 *in vitro* (207 μM final concentration of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 16 mM final concentration of TRITON X-100) according to the present method (curve a) with an absorption spectrum of a commercially prepared MTT-formazan dissolved in DMSO (curve b) as constructed from peak absorption data reported in Plumb et al., Cancer Research, 49:4435-4440; Kasugai et al., Japan Journal of Pharmacology, 52:95-100; and Carmichael et al. mentioned above.

Fig. 2 shows a comparison of the absorption spectrum (curve a) of Fig. 1 compared to an absorption spectrum of commercially prepared MTT-formazan dissolved in isopropanol (curve c) as constructed from peak absorption data reported in Denizot et al., Journal of Immunological Methods, 89:271-277.

Fig. 3 shows a comparison of the absorption spectrum (curve a) of Fig. 1 compared to an absorption spectrum of commercially prepared MTT-formazan dissolved in TRITON X-100 (curve d).

Fig 4. shows a graphical representation of a comparison of the present method for determination of the number of *E. coli* cells and the plate count method.

Detailed Description of the Invention

Use of a nonionic detergent solubilizer for formazan compounds in a tetrazolium assay method permits *in vitro* monitoring of a cell metabolism without substantially damaging the cell. Thus, the cell kinetic response to both growth and cytotoxic factors can be quickly assessed. The method of the present invention can be used, for example, to determine bacteria sensitivity to antibacterial agents, mammalian cell sensitivity to anticancer agents or the efficacy of growth factors used in the field of biotechnology. In comparison to the prior art, rapid results and simple procedures make the present invention conducive for space use.

Cells monitored by the present method are preferably cultured in a suitable aqueous nutrient medium. The nutrient medium contains components necessary for sustaining cell growth. Such nutrient media or broths are known in the art. Examples include Mueller-Hinton broth as recommended by the National Committee for Clinical Lab Standards, trypticase soy broth, and the like. The growth medium typically comprises dextrose and other sugars to promote rapid growth or replication of the cell species.

Cells useful in the present method include microorganisms such as bacteria, fungi, protozoa and helminths and mammalian cells taken, for example, from tissue biopsies, donor animals, and the like. Depending on the application, the cells can be obtained from a variety of locations. The American Type Culture Collection (ATCC) is a source for standard commercially available strains useful, for example, in screening the efficacy of a drug or antiseptic. Mammalian cells are also cultured commercially as ATCC standard stocks. The microorganism cells can be isolated and cultured from

streams in water treatment processes where the cells are present as contaminants, or from biological fluids such as urine, stool suspensions, blood, spinal fluid, tissue suspensions, and the like where the cells are present as pathogens.

The indicator component in the present method is a tetrazolium compound which permits chromogenic determinations of cell metabolism. Suitable tetrazolium compounds are relatively colorless in an unreduced state, but are reduced to a formazan dye in a reduced state. Such compounds are particularly useful because the formazan is made by a cell at a consistent uniform rate. While it has been found that further reduction reactions involving the formazan compounds can result in other products which are colorless, these reactions are generally not significant during the time frame of the present method.

Since certain tetrazolium compounds can potentially be toxic to some types of microorganisms at a given concentration, suitable tetrazolium compounds should be substantially innocuous at a concentration useful for producing a visually detectable color change.

Examples of suitable tetrazolium compounds include 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) commercially available under the trade designation RED TETRAZOLIUM, 2,5-diphenyl-3-(α-naphthyl)-1-tetrazolium chloride commercially available under the trade designation TETRAZOLIUM VIOLET (TV), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT), 3,3'-(4,4'-biphenylene)bis(2,5-diphenyl-2H-tetrazolium chloride commercially available under the trade designation NEOTETRAZOLIUM, 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis(2,5-diphenyl-2H-tetrazolium chloride commercially available under the trade designation BLUE TETRAZOLIUM, 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis(2-(4-nitrophenyl)-5-diphenyl-2H-tetrazolium chloride)

commercially available under the trade designation NITROBLUE TETRAZOLIUM, 3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis(2,5-bis(4-nitrophenyl)-2H-tetrazolium chloride) commercially available under the trade designation TETRANITROBLUE TETRAZOLIUM, and the like. Other tetrazolium compounds can also be obtained from various suppliers, such as, for example, Sigma Chemical of St. Louis, MO or Polysciences of Warrington, PA. The most preferred tetrazolium compound is MTT. MTT has been found to have a minimal toxic effect on cell growth and also obtains a distinct purple color upon reduction to the formazan constituents.

The tetrazolium compound is typically used in the present method in an amount of at least about 1 μM and preferably in an amount from about 20 μM to about 250 μM.

The color developing component of the present method is a mild nonionic detergent or surfactant. Such detergents have been found to solubilize and/or homogeneously disperse hydrophobic formazan compounds which are ordinarily insoluble in the aqueous test medium. Furthermore, such detergents are substantially innocuous to most cell types at low concentrations and can therefore be used *in vitro* (at appropriate concentrations) without substantially adversely affecting the growth in the culture. Thus, the color change can be followed in a fashion which is neither substantially interfering nor destructive to the cell culture.

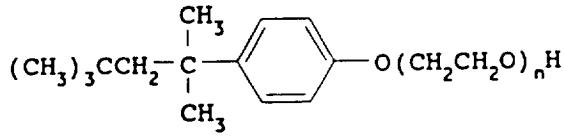
Choice of detergent depends on formazan solubilizing sensitivity and degree of toxicity to a given cell type. It is known that microorganism cells are more resistant to a detergent than mammalian cells and that injured cells are more sensitive to a detergent than uninjured cells. In general, however, it has been found that cell disruption can be substantially avoided in the present method while maintaining required formazan sensitivity. This is due in part at least to the high sensitivity of the detergent to the formazan product. It is believed that competing hydrophobic formazan molecules in the test

medium are preferentially bound to the nonpolar monomer sites in the detergent molecule.

Overall, variables important in detergent selection include speed at which the formazan-detergent aggregates are formed, critical micelle concentration, intensity of the developed color, influence on cells with membrane injury and sensitivity of the formazan absorption spectrum to changes in cell number, nominal variations in pH and variations in volume of the growth medium.

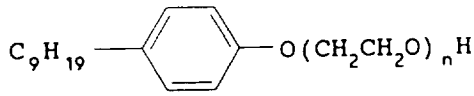
Examples of classes of nonionic detergents are alkylene oxide ethers of phenols, fatty acids and alkyl mercaptans; alkylene oxide esters of fatty acids, alkylene oxide ethers of fatty acid amides; the condensation product of alkylene oxide with partial fatty acid esters, and the like wherein the polyoxyalkylene chain can contain from 4 to about 30 alkylene repeat units and the alkylene unit has 2 or 3 carbon atoms.

A preferred class of nonionic detergents includes polyethylene oxide ethers of para C_8 or C_9 alkylphenol exemplified by octoxynol and nonoxynol. Octoxynol has the formula:



wherein n is the number of ethylene oxide repeat units and can vary from about 5 to about 15. Also known as polyethylene glycol p-isooctylphenyl ether and octylphenoxy polyethoxyethanol, octoxynol where n is 9 to 10 is commercially available under the trade designations CONCO NIX-100, IGEPAL CA-630, NEUTRONYX 605 and TRITON X-100. TRITON X-100 is a tradename of Rohm & Haas. Octoxynol (N.F.) is a mixture wherein n ranges from 5 to 15 with an average of 10. Octoxynol-9 has an average composition of n=9.

Nonoxynol has the formula:



wherein n is the number of ethylene oxide repeat units and can vary from about 4 to about 30. Nonoxynol is commercially available under the tradenames CONCO NI, DOWFAX 9N, IGEPAL CO, MAKON, NEUTRONYX 600'S, NONIPOL NO, POLYTERGENT B, RENEX 600'S, SOLAR NP, STEROX, SURFONIC N, T-DET-N, TERGITOL NP and TRITON N.

Specific representative examples of other suitable nonionic detergents include n-dodecyl-β-D-maltoside (DM), n-dodecyl-β-D-gluco-pyranoside (DP), diethyleneglycol monopentyl ether (DE), polyethoxyethanol lauryl ether, polyoxyethanol sorbitan monolaurate commercially available under the trade designation TWEEN-20, polyethylene glycol fatty alcohol ether commercially available under the trade designation TERGITOL-15-S-30, polyoxyethanol sorbitan monooleate commercially available under the trade designation TWEEN-80 and polyethylene glycol monododecyl ether commercially available under the trade designation LUBROL PX and nonionic detergents commercially available as NP-35 and NONIDET-P40. In the present method, octoxynol/TRITON X-100 is a most preferred detergent.

The detergent is generally used in an amount of from about 0.5 mM to about 400 mM, preferably from about 1 mM to about 20 mM.

An electron carrier is an optional but preferred component of the present method to facilitate tetrazolium salt reduction by the cells. The electron carrier transports electrons used in the redox reaction and can enhance the speed and color intensity of the color change. Examples of suitable electron carrier compounds include N-methylphenazonium methosufate (PMS) commercially available from Polysciences, N-ethylphenazonium ethosulfate commercially available from ICN Biochemicals of Plainview, NY and phenazine N-oxide

also commercially available from ICN Biochemicals. A preferred electron carrier is PMS.

The electron carrier is typically used in an amount of from 0 to about 600 μM and preferably used in an amount of from about 100 μM to about 400 μM.

The present method preferably comprises an *in vitro* indicator composed of MTT, octoxynol (TRITON X-100) and PMS. This indicator was found to have a relatively high sensitivity and compatibility for both microorganism and mammalian cells.

In many applications, the method will include an addition of an effector agent such as antimicrobial (including both chemotherapeutic and antiseptic), antibiotic, anticancer agents and combinations thereof for efficacy assessment. Other agents can include growth factors and chemical materials and waste streams for environmental toxicity testing. In addition, a substrate material can be added to test for biodegradability.

Specific representative examples of antiseptic antimicrobial agents include iodine, hydrogen peroxide, chlorine, benzalkonium chloride, hexachlorophene, and the like. Specific representative examples of chemotherapeutic antimicrobial agents include isoniazid; ethambutol; sulfonamides such as sulfamethoxazone, para-aminobenzoic acid, sulfanilamide, and the like; nitrofurans such as nitrofurantoin, nitrofurazone, nifuroxine, and the like; trimethoprim; and nalidixic acid. Specific representative examples of antibiotics include natural penicillin; semisynthetic penicillins such as ampicillin, methicillin, oxacillin, and the like; aminoglycosides such as streptomycin, neomycin, gentamicin; cephalosporins such as cephalothin, cefamandole, cefotaxime and moxalactam, and the like; tetracyclines; chloramphenicol; macrolides such as erythromycin; polypeptides such as bacitracin; vancomycin; rifamycins such as rifapin; and the like. Specific representative examples of antifungal agents include polyenes such as nystatin, amphotericin B, and

the like; imidazoles such as ketoconazole, miconazole, and the like; and griseofulvin. Specific representative examples of antiprotazoan and antihelminthic agents include, quinine, chloroquine, emetine, metronidazole, niclosamide, piperazine, and antimony compounds.

The effector agent will typically be used in an amount which varies based on the number of cells in the test and an estimated power of the agent involved.

Since the metabolism of both microorganism and mammalian cells can now be monitored *in vitro* without substantially impairing cell activity, the present method has use in any biologic application wherein an extent of cell proliferation in various environments is important knowledge: The number of cells present in a pure culture can be accurately estimated from the time required for the appearance of color. This is based on the fact that cells of the same variety produce similar amounts of dehydrogenase.

Agents which either inhibit or stimulate cell growth (i. e. effector agents) can be rapidly screened. The effectiveness of antimicrobial disinfectants, antibiotics and anticancer agents can be determined. In addition, microorganism resistance to such agents can be detected as well as genetic alterations in Ames test microorganisms. As another related application, the environmental toxicity of chemical substances or waste products can be gauged (i. e. the degree to which the product adversely affects cell metabolism).

Agents which enhance cell growth have numerous applications in environmental, agricultural and biotechnology fields. Bacterial species containing genes inserted by recombinant DNA techniques are cultured in bioreactors wherein expression of the artificial gene produces a desired product. Cultured yeasts and other microbes have a variety of food producing and waste disposing applications.

As a further application, the present method can be used in screening materials for biodegradability (i. e.

the degree to which the material (as substrate) sustains microorganism cell growth).

The present method is conveniently carried out in a conventional 96 well microtiter plate wherein the well generally has a volumetric capacity of 250 μL. Multiple repetitions are preferably used so that the results can be evaluated statistically as well as quantitatively. Each microplate permits 96 assays to be conducted simultaneously. It is recognized that culture plates having a greater or fewer number or wells could also be used.

Typically a source of cells is obtained either from a stock culture grown on a medium such as agar using conventional techniques or from a sample of bodily fluid to be tested. If the cells are grown in a stock culture, they can be swabbed into suitable nutrient broth or sterile reagent grade water for preparing cell dilutions having an appropriate concentration. Solutions of MTT, TRITON X-100, and PMS having an appropriate concentration can also be prepared by diluting stock solutions of the reagents. Each assay generally comprises from about 10^5 to about 10^8 cells.

The microtiter plates are then prepared (in triplicate or however many replications are desired) by adding to each well a sufficient amount of nutrient broth, cell suspension, antimicrobial/effector agent to be tested, if appropriate, and the indicator solutions. The plate is shaken for a short period of time (30 seconds is typically adequate) and then placed in an incubating environment at a suitable temperature for a sufficient amount of time to obtain a color indication. Color change over time can be evaluated using a spectrophotometric analyzer combined with an incubator to obtain kinetic plate readings. An $Thermo_{max}$ microplate reader commercially available from Molecular Devices Corp. of Menlo Park, Ca. is a suitable device.

While the exact test duration (incubation time) will vary based upon the concentration of the indicator

reagents used and the type and quantity of cells per well, the test duration will typically be 20 to 120 minutes and is subject to practitioner preference.

The present method is further illustrated by the following examples:

Examples

Microorganisms

Escherichia coli and *Citrobacter freundi* bacteria were isolated from the tap water distribution system of the City of Houston, Tx. and provided by the Water Quality Control Div.

Bacillus lichenformans were isolated from the space shuttle (STS 48) potable water system.

Two *Pseudomonas cepacia* strains used were isolated from the space shuttle potable water system of STS 39 and STS 48 and another was commercially obtained (ATCC 49129).

Candida albicans (ATCC 14053) were commercially obtained.

The microorganisms were maintained on standard methods agar (SMA) unless otherwise noted. *C. albicans* was maintained on sabouraud dextrose agar (SAB). Agar media were obtained from BBL Microbiological Systems of Cockeysville, Md.

For each assay, the microorganisms were freshly cultured on the agar plates at 30°C. Two or three hours prior to the assay incubation temperature was raised to 35°C. Test microorganisms were swabbed off the agar surface and suspended in sterile reagent grade water (Milli Q water system) obtained from Millipore Corp. of Bedford, Ma, to a turbidity of OD_{590nm}=1.0. This density was found equivalent to $2x10^9$ *E. coli*, *B. lichenformans*, and *P. cepacia* using the spread plate technique given in American Public Health 17th ed. From this value, cell amounts for dilutions were extrapolated.

Mammalian Cells

Mammalian cells comprised CESS human lymphoblastic cells, (ATCC TIB 190).

The mammalian cells were maintained in RPMI-1640 comprising 25 mM Hepes buffer and L-glutamine obtained from Whittaker Bioproducts. The RPMI-1640 was supplemented with 10 wt % fetal bovine serum (FBS) obtained from Gibco Laboratories of Grand Island, NY and 40 μg/ml of gentamicin obtained from Sigma Chemicals. The cells were incubated for 24 hours at 37°C in 5 vol % CO_2. Viable cell number was determined before each experiment by a trypan dye exclusion technique (trypan blue 0.4%) using a hemocytometer.

All trials used cultures in the exponential growth phase.

Reagents

Reagents used in the examples including the tetrazolium compounds, detergents, PMS, dyes, antibiotics and antiseptics were typically obtained from Sigma Chemical unless otherwise noted.

Tetrazolium Assay Procedure

For microorganisms, the tetrazolium assay was run in triplicate using 96 well microtiter plates. Cell dilutions having a turbidity reading of OD_{590nm}=0.6 were prepared from the more concentrated stock suspension. Typically, an aliquot of 25 μL of the diluted cell suspension was added to each well followed by a 50μL aliquot of double strength trypticase soy broth. Next, the following aliquots were added to the wells: 15 μL of 1.2 mM MTT; 10 μL of 0.06 mM PMS; and 25 μL of 160 mM TRITON X-100. The plate was mixed for 30 seconds on a Hyperion plate shaker from Fisher Scientific and placed into the $Thermo_{max}$ microplate reader for incubation and analysis of the formazan production. The plates were

incubated at 30°C. The formazan production was followed spectrophotometrically over time using a test wavelength of 590 nm and a reference wavelength of 650 nm. A background control comprising an identical plate minus the cells was also run to account for spontaneous changes in the optical density.

Assay of mammalian cells was similar to that of microorganism cells except that 150 μL of mammalian cell dilution ($5x10^5$ cells) in RPMI 1640 was used and the incubation temperature was 37°C.

For each assay test, negative controls were conducted to determine whether tetrazolium reduction was caused by factors other than cell metabolism during the test run. Controls were conducted for the presence of residual dehydrogenase enzyme from the stock culture and for the influence of other reagents on tetrazolium reduction.

The control for residual enzyme was run by conducting the tetrazolium assay (as described above) on freshly killed cells. The cells were killed using iodine. The amount of iodine required was determined by the plate count method. The control for the influence of other reagents on tetrazolium reduction was run by conducting assays containing only the tetrazolium compound and the reagent to be tested. The controls indicated that outside influence on the final spectrophotometric determination for formazan was less than about 2.5 %.

Examples 1 and 2

Cell Concentration vs. Formazan Production for *E. Coli* and CESS

Tetrazolium assays according to the above procedures were performed on *E. coli* (Example 1) and CESS cells (Example 2) wherein cell amounts on microtiter plates varied between 10^5 and 10^7 cells/well. The formazan production was followed over time. The final concentration of TRITON X-100 was 16 mM. The *E. coli* assay time was 45 min and the CESS assay time was 2 hr.

Results are given in Table 1. Graphical representations of the data show that formazan production as determined by spectrophotometric analysis was linearly proportional to cell numbers with an $r^2=0.99$ for *E. coli* and $r^2=0.98$ for CESS. The data also suggest that 10^7 cells/well give an optimal assay time of from 30-45 minutes for microorganism cells. As a control, plates containing 10^7 cells/well were incubated at room temperature to show that carry over metabolism (from the stock culture) was not responsible for the formazan production observed (but rather production was due to growth under the prescribed conditions).

Table 1

Example 1 (E. coli)		Example 2 (CESS)	
Conc. Cells per Well ($x10^6$)	Absorbance* (OD @ 590-6590 nm)	Conc. Cells per Well ($x10^5$)	Absorbance* (OD @ 590-6590 nm)
10.5	0.33	10.5	0.34
20.9	0.68	20.9	0.68
31.3	1.04	31.3	1.03
41.3	1.44	41.8	1.43

*-Each data point represents the average of 8 wells plus or minus the standard deviation.

Example 3 and Comparative Examples 1-3

Comparison of *in vitro* TRITON X-100 with Post-incubation Solvents and TRITON X-100

In the following example and comparative examples, formazan solubilizing capacity of *in vitro* TRITON X-100 of the present invention was compared to DMSO and isopropanol solvents and TRITON X-100 added post incubation.

Tetrazolium assays of *E. coli* were conducted as described above, except that in the comparative examples, TRITON X-100 detergent was either omitted or added at the end of the incubation period. Capacity was based on color intensity determined by spectrophotometric absorbance at 590-650 nm. The assay time was 30 min, the final concentration of TRITON X-100 was 16 mM and the amount of DMSO or isopropanol added was 120 μL.

Results shown in Table 2 indicate the superior developing efficiency of TRITON X-100 especially when added *in vitro* as compared to DMSO and isopropanol.

Table 2

Example No.	Developer	Absorbance (O.D. (590-650 nm))
3	TRITON X-100 (in vitro)	0.77
Comp. 1	TRITON X-100 (post-incubation)	0.66
Comp. 2	DMSO	0.23
Comp. 3	isopropanol	0.17

Examples 4-36

Comparison of Nonionic Detergents for *In vitro* Formazan Solubilizing

Tetrazolium assays of *E. coli* cells were conducted *in vitro* as described above except that the tetrazolium compound, detergent type and detergent concentration were varied. Results are given in Table 3. (One weight percent is approximately 16 mM final concentration.)

Table 3

Example No.	Detergent	Detergent Conc. (wt %)	Tetrazolium Compound	Absorbance[e]
4	DP	1.0	MTT[a]	<0.1
5	DP	0.1	MTT	<0.1
6	DM	1.0	MTT	0.88
7	DM	0.1	MTT	0.13
8	DE	1.0	MTT	<0.1
9	DE	0.1	MTT	<0.1
10	TRITON X-100	1.0	MTT	1.11
11	TRITON X-100	0.1	MTT	0.65
12	DP	1.0	INT[b]	0.28
13	DP	0.1	INT	0.23
14	DM	1.0	INT	0.43
15	DM	0.1	INT	0.39
16	DE	1.0	INT	0.11
17	DE	0.1	INT	0.25
18	TRITON X-100	1.0	INT	0.61
19	TRITON X-100	0.1	INT	0.42
20	DP	1.0	TTC[c]	<0.10
21	DP	0.1	TTC	<0.10
22	DM	1.0	TTC	0.15
23	DM	0.1	TTC	0.10
24	DE	1.0	TTC	<0.10
25	DE	0.1	TTC	<0.10
26	TRITON X-100	1.0	TTC	0.35
27	TRITON X-100	0.1	TTC	0.25
28	DP	1.0	TV[d]	0.15
29	DP	0.1	TV	<0.10
30	DM	1.0	TV	0.32
31	DM	0.1	TV	0.15
32	DE	1.0	TV	<0.10
33	DE	0.1	TV	<0.10
34	TRITON X-100	1.0	TV	0.72
35	TRITON X-100	0.1	TV	0.61

a-Absorbance measured at 590-650 nm after 2 hr.
b-Absorbance measured at 450-650 nm after 2 hr.
c-Absorbance measured at 450-650 nm after 24 hr.
d-Absorbance measured at 590-650 nm after 24 hr.
e-Absorbance reading represents an average of 3 wells plus or minus the standard deviation.

Although the detergents tested had a similar critical micelle concentration and molecular weight, various degrees of formazan dispersion were observed. TRITON X-100 was the most effective detergent and TRITON X-100/MTT was the most effective indicator combination.

Examples 36 and 37

Formazan Metabolization versus Incubation Time

In the following examples, tetrazolium assays *of E. coli* cells were conducted *in vitro* as described above for microorganisms except that the final TRITON X-100 concentration was reduced from 16 mM (Example 36) to 1.6 mM (Example 37) and the spectrophotometric evaluation was continued for 210 min. The number of cells per well was about 2.5×10^7. Results are given in Table 4.

Table 4

Time (min)	Absorbance* (O.D. a 590-650 nm)	
	Example 36	Example 37
20	0.36	0.32
40	0.63	0.6
100	1.58	1.3
130	1.42	0.77
210	1.65	0.36

*-Each absorbance represents an average of 8 wells plus or minus the standard deviation.

Data in Table 4 show that formazan was further reduced by cell metabolism over time to a colorless product. This was demonstrated by lowering TRITON X-100 concentration from 16 mM (1 wt %) to 1.6 mM (0.1 wt %). A possible explanation for this result is that in the higher concentration run, a sufficient amount of monomer sites which bind formazan were present and in the low concentration run, the formazan was only partially bound allowing further reduction to the colorless state.

Example 38 and Comparative Examples 4 and 5

Comparison of the Absorbance Spectra of MTT-Formazan in TRITON X-100 to MTT-Formazan in Other Solvents

For the following examples and comparative examples, a tetrazolium assay *of E. coli* cells was conducted *in vitro* as described above with a final TRITON X-100 concentration of 16 mM and a final MTT concentration of

207 μM. The spectrograph from this assay was obtained (spectrum (a) in Figs. 1-3). This spectrograph was overlaid with spectrographs constructed from peak absorption data obtained from the references Plumb et al., Kasugai et al., Carmichael et al., and Denizot et al. for a commercially produced MTT-formazan (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-formazan (from Sigma Chemical) (207 μM)) dissolved in DMSO, (spectrum (b) in Fig. 1, Comparative Example 4); isopropanol (spectrum (c) in Fig. 2, Comparative Example 5); and TRITON X-100 (spectrum (d) in Fig. 3, Example 38).

The absorbance peaks of the MTT-formazan in DMSO (Fig. 1(b)), isopropanol (Fig. 2(c)), and TRITON X-100 (Fig. 3(d)) were very different. However, the commercial formazan dispersed in the TRITON X-100 (Fig. 3(d)) had an identical absorbance peak as the MTT-formazan produced by *E. coli* and dispersed *in vitro* with TRITON X-100 (Fig. 3(a)). This is evidence that use of TRITON X-100 gives a stable absorption spectrum which is relatively unaltered by assay conditions.

Example 39 and Comparative Example 6

Sensitivity of *E. coli* to Iodine Inhibition

An *E. coli* strain (City of Houston) was contacted with iodine and then assayed using the present tetrazolium method to determine the susceptibility of these bacteria to iodine inhibition. Fresh iodine stock solutions were prepared in reagent grade water by the N,N-diethyl-p-phenylenediamine colorimetric method described in American Public Health 17th ed. using a DR/2 spectrophotometer commercially available from Hach Co, of Loveland, Co. so that a final concentration of each solution was between 2-20 ppm iodine. *E coli* cells maintained as described earlier were resuspended in sterile reagent grade water to a concentration of OD_{590nm}=0.6 then diluted again 1 part cells to 2 parts water. Aliquots (50 μL) of both the iodine stock

solution and cell suspension were placed in the wells for a contact time period of 10 min at 25°C. At the end of this time, the iodine was neutralized with 10 μL of 0.02mM of sodium thiosulfate. The amount of sodium thiosulfate required for neutralization was determined from a dose response curve showing the minimum concentration needed to neutralize 40 ppm iodine. The plate was mixed for 30 seconds after which 50μL of the soy broth was added followed by 15 μL of 1.2 mM MTT, 10 μL of 0.06 mM PMS and 25 μL of 160 mM of TRITON X-100. The plates were again shaken for 30 seconds and incubated at 30°C. The effect of the iodine on inhibiting cell growth was determined by comparing the reduction in absorbance of the dosed cells against a control population not dosed with iodine. The results are given in Table 6.

The iodine sensitivity of the same *E. coli* cells was also determined by a plate count assay. A comparison of the data represented in Fig. 4. Both method showed that the number of cells was reduced linearly with increasing iodine concentration. There was also no significant difference in the curve slopes for each method ($p=0.84$, $n=6$) establishing the present tetrazolium method as a valid method for enumerating relative numbers of viable cells following iodine exposure.

Table 6

Iodine Conc. (ppm)	Inhibition (%)			
	Ex. 39 E coli	Ex. 40 P. cepacia (ATCC 49129)	Ex. 41 P. cepacia (STS 39)	Ex. 42 P. cepacia (STS 48)
0	0	0	0	0
1	13.1	6.7	0	8.6
2	37.4	14.3	0	10.0
3	73.7	37.6	6.2	20.1
4	96	54.8	11.2	24.2
5	100	72.6	17.5	44.5
6	-	100	27.5	48.8
7	-	-	42.5	68.9
8	-	-	56.2	81.8
9	-	-	71.7	100
10	-	-	100	

Examples 40-42

Iodine Inhibition of Space Shuttle Isolates

In the following examples, the effect of iodine disinfectant on bacteria isolates (*P. cepacia*) taken from the potable water system from two space missions was determined by the tetrazolium assay method described in Example 39 and Comparative Example 6. In addition, commercially obtained *P. cepacia* (ATCC 49129) were run as a control to determine the acquired resistance of the space shuttle strains to iodine. Results are given in Table 6. It can be seen that the strains taken from the space shuttle had acquired a significant resistance requiring an iodine concentration as much as a 67 % greater for the strain from STS 39 (Example 41) compared to the stock strain (Example 40).

Examples 43-46

Benzalkonium Chloride Inhibition

In the following examples, the present tetrazolium assay method was used to determine the percent viability

of several bacteria strains in the presence of benzalkonium chloride (BAC). Data are given in Table 7.

Table 7

Benzalkonium Chloride (ppm)	Bacteria viability (%)			
	B. lichenformans (STS 48)	E. coli (City of Houston)	Citrobacter freundi (City of Houston)	Candida abicans (ATCC 14053)
0	100	100	100	100
2	91.6	89.1	88.6	-
3	66.6	47.8	32.8	50.5
4	38	14.1	15.7	-
5	0	0	0	0

Example 47-53

Reduction of Benzalkonium Chloride Inhibition of E. coli in the Presence of Various Metal Cations

In the following examples, *E. coli* cells were contacted with 3 ppm of benzalkonium chloride (BAC) and varying concentrations of a variety of metal cations and then assayed using the present tetrazolium method to determine the reduction of antibiotic properties (if any) of BAC in the presence of the cations.

For the various cations, 200 mM stock solutions of $AlCl_3$, $SrCl_2$, $BaCl_2$, $CaCl_2$, $MgCl_2$, KCl and NaCl metal chlorides were initially prepared. These solutions were then diluted to a concentration on the order of 0.4 mM. Aliquots of the cation stock solution were added to the wells containing *E. coli* cells (50 μL, OD_{590nm}=0.6) and BAC to give the desired cation concentration and the microplates were shaken for 30 sec. The soy broth and tetrazolium assay solutions were added to the wells as described previously and the microplate was shaken again for 30 sec. The cell metabolism was followed using spectrophotometric anaylsis. The percent reduction in inhibitory activity of BAC caused by the cations was determined by comparison to control wells containing BAC but no cations. Results are given in Table 8.

Table 8

Example	Cation Type	Inhibition (%, non-normalized)								
		Cation Concentration(μM)								
		0	0.0003	0.0005	0.0008	0.0010	0.0025	0.0050	0.0075	0.0100
47	Al^{+3}	64.2	42.6	34.7	18.3	14.8	9.9	10.4	12.2	2.3
48	Sr^{+2}	59.4	43.8	23.3	15.7	14.9	3.9	8.3	13.6	9.2
49	Ba^{+2}	56.8	53.2	51.9	45.2	36.7	22.4	14.0	3.8	6.8
50	Ca^{+2}	49.3	47.7	44.9	38.5	38.2	28.4	14.1	8.2	11.0
51	Mg^{+2}	59.2	52.5	52.9	46.2	45.5	38.0	34.4	25.4	13.0
52	K^{+}	58.9	55.5	57.3	57.3	53.3	60.4	55.1	64.6	48.9
53	Na^{+}	59.7	57.6	56.3	54.6	47.4	55.8	60.4	58.9	45.7

It can be seen from the data that increasing concentrations of cations reduced the effectiveness of BAC inhibition activity. However, the affect of increasing K^{+} and Na^{+} cation concentration was much less pronounced indicating that the cation valence was an important factor.

Examples 54 and 55

Effect of TRITON X-100 on Injured *E. Coli*

In the following examples, the effect of TRITON X-100 concentration on *E. coli* cells damaged by iodine exposure was examined. Using the procedures outlined in Example 39 and Comparative Example 6, wells were established containing iodine injured and non-injured *E. coli* cells. To some of the injured cells, tetrazolium assay reagents were added wherein the TRITON X=100 strength was either 16 mM (1 wt %) or 1.6 mM (0.1 wt %). In addition, reference wells containing only normal (non-injured cells) were set up as well as control wells containing injured cells without tetrazolium reagents and normal cells with tetrazolium reagents (both 1.0 and 0.1 wt % TRITON X-100). The wells were then incubated for 40 min at 30°C as for a normal tetrazolium assay. Following the incubation period, viable cells were enumerated using the plate count method by swabbing from the wells to m-T7 agar. The level of injury was reported as the percent difference in the plate counts between the reference or

control wells without TRITON X-100. The results are given in Table 9.

Table 9

Example	TRITON X-100 (wt %, final)	Difference in plate count based on reference or control plate (%)			
		Concentration Iodine (PPM)			
		0	2	3	4
Ref./Control	0	100	74.3	26.2	1.6
54	0.1	96.2	76.5	20.2	1.6
Ref./Control	0	100	89.7	56.6	13.4
55	1.0	94.2	57.1	30.7	18.8

It can be seen that the TRITON X-100 at 1.0 wt % had a large negative effect on the viability of iodine damaged cells compared to the control. However, at the lower concentration, the TRITON X-100 detergent had little negative effect on the cells.

The foregoing description of the biotechnical assay method is illustrative and explanatory thereof. Various changes in the reagents, apparatus, and particular techniques employed will occur to those skilled in the art. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

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Abstract of the Invention

A method for conducting an *in vitro* cell assay using a tetrazolium indicator is disclosed. The indicator includes a nonionic detergent which solubilizes a tetrazolium reduction product *in vitro* and has low toxicity for the cells. The incubation of test cells in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and octoxynol (TRITON X-100) permits kinetics of the cell metabolism to be determined.

MSC-21979-1

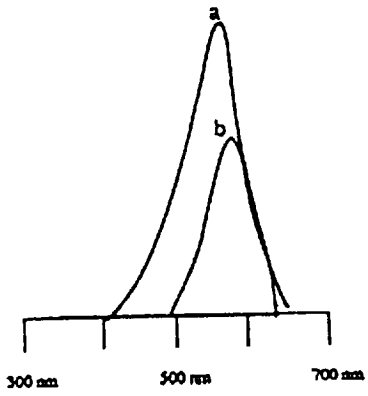
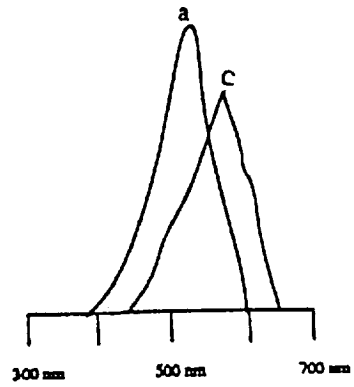


Fig. 1



MSC-21979-1 Fig. 2

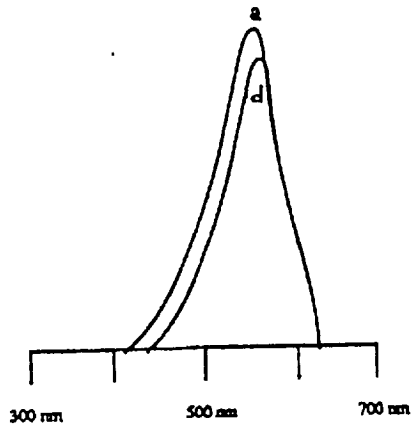


Fig. 3

MSC-21979-1

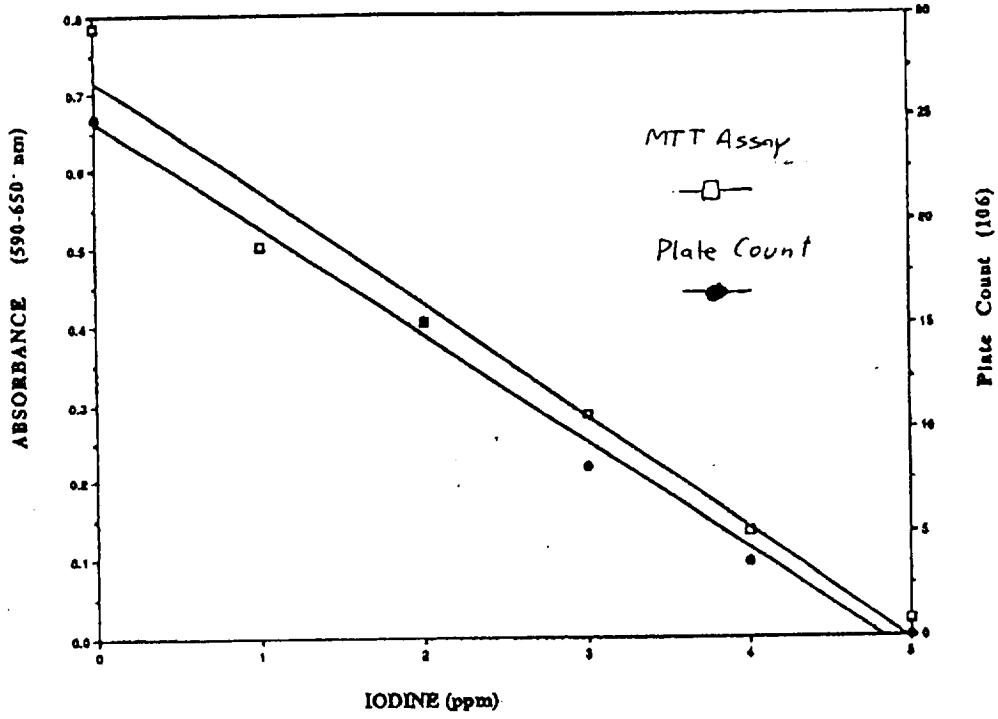


Fig. 4